Spring 5-3-2022

Role Of Fasting In Caloric Restriction Improved Glucose Tolerance

Makayla M. Dillon
Cleveland State University

ROLE OF FASTING IN CALORIC RESTRICTION IMPROVED GLUCOSE TOLERANCE

MAKAYLA DILLON

Bachelor of Biology

Ohio University

May 2020

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE IN BIOLOGY

at the

CLEVELAND STATE UNIVERSITY

May 2022

We hereby approve this thesis for

MAKAYLA DILLON

Candidate for the Master of Science in Biology Degree

for the Department of Biological, Geological and Environmental Sciences

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies by

Dr. Roman Kondratov, Major Advisor
GRHD/BGES, Cleveland State University Date: 05/03/2022

Dr. Crystal M. Weyman, Thesis Committee Member
GRHD/BGES, Cleveland State University Date: 05/03/2022

Dr. Kailash Gulshan, Thesis Committee Member
GRHD/BGES, Cleveland State University Date: 05/03/2022

Student's Date of Defense: 05/03/2022

DEDICATION

This work is dedicated to my family. I'm fortunate to have received the encouragement they have given me during this program. As a first generation student, it is a privilege to have the opportunity to complete a graduate degree and I am very thankful.

ACKNOWLEDGEMENTS

I'd like to first acknowledge Dr. Kondratov for his mentorship, patience and guidance. I am very fortunate to have had him as my advisor. I will always value how supportive he was to me in the program and beyond it, as he fully supported me in my application to dental school while working on this project. A labmate of mine, Artem, mentioned that Dr. Kondratov is always two steps ahead– and it is true. He truly teaches us forward and critical thinking when we have trouble with this. I greatly appreciate the time he took to help me prepare for any presentations, revising my work, and guiding me through my project and the completion of this program.

I'd like to next acknowledge my labmates Artem, Olga, Patrick, Allan, and Jonathan. Their willingness to help me in all aspects of my project is very appreciated and never went unnoticed. Thank you to all of my labmates for guidance through courses, help with experiments, interpretation of results, fine-tuning of presentations, and help with administrative business. A special thank you to Artem, whose mentorship continuously helped me through the program. I wish you all good luck in completing your programs and all the best wishes for you and your families.

I am incredibly grateful for the encouragement, love, and support shown by my family and friends during this program. In particular I'd like to acknowledge my partner, Joseph, whose support was essential to my success in the program. As a student in the same discipline, his support in all aspects of this program was invaluable. I'm very grateful for his help with everything from courses, to data analysis, to helping me with my thesis, and so much more.

ROLE OF FASTING IN CALORIC RESTRICTION IMPROVED GLUCOSE TOLERANCE

MAKAYLA DILLON

ABSTRACT

The Caloric Restriction (CR) diet in mammals has been shown to increase longevity and prevent metabolic disorders. Some of the benefits of CR include improved glucose homeostasis, namely improvements in glucose tolerance. Mice on the CR diet have a 30% reduction in food provided, as well as a fasting period between meals as they are only fed once a day. Hence the benefits of CR stem from both fasting and reduced calorie intake. In this study, we investigated if the fasting component of CR is responsible for the benefits of this diet in improving glucose homeostasis. In particular, we used glucose tolerance tests to show that varying durations of fasting affect the glucose tolerance of fasted mice differently. Shorter fasts improved glucose tolerance, while longer fasts showed similar or reduced tolerance compared to non-fasted mice. Ultimately, we observed that fasting may be responsible for a part of the benefits of CR, but it is not the only contributor to the advanced glucose tolerance and homeostasis of mice on CR.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	Adenosine Di-Phosphate
AKT	Activated Protein Kinase B
AMPK	AMP-activated protein kinase
AL	Ad-libitum
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
CR	Calorie Restriction
CVD	Cardiovascular Disease
dL	Deciliter
ELISA	Enzyme-Linked Immunosorbent Assay
FFA	Free Fatty Acid
G1P	Glucose-1-Phosphate
G6P	Glucose-6-Phosphate Dehydrogenase
GLUT2	Glucose Transporter Type 2
GLUT4	Glucose Transporter Type 4
GK	Glucokinase
GP	Glycogen Phosphorylase
GPCR	G-protein Coupled Receptor

GS	Glycogen Synthase
GSK3	Glycogen Synthase Kinase 3
GTT	Glucose Tolerance Test
HDL	High Density Lipoprotein
HK	Hexokinase
IACUC	Institutional Animal Care and Use Committee
IF	Intermittent Fasting
IGF-1	Insulin-like Growth Factor 1
IP	Intraperitoneal
IRS	Insulin Receptor Substrate
LD	Light/Dark cycle
mg	milligram
mL	milliliter
mTOR	mammalian Target of Rapamycin
mTORC	mammalian Target of Rapamycin Complex
NIH	National Institute of Health
PDK1	Pyruvate Dehydrogenase Kinase 1
PEPCK	Phosphoenolpyruvate Carboxykinase
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PIP3	Phosphatidylinositol 4,5-Triphosphate
PPP	Pentose Phosphate Pathway
SEM	Standard Error of Mean

SF	Spontaneous Fast
SNARE	SNAP Receptor
TRF	Time-Restricted feeding
ZT	Zeitgeber Time

CHAPTER I

INTRODUCTION

The prevalence of negative diseases attributed to metabolic imbalance, namely disruption in glucose homeostasis, in the United States is staggering and rapidly increasing. Metabolic imbalance encompasses a plethora of various health conditions that often accumulate, leading to the development and progression of potentially life threatening pathologies. The most alarming of pathologies being that cardiovascular disease (CVD) is the leading cause of death in the United States, one person dying every 36 seconds from the disease (CDC, 2022). These health conditions have a broad, and overall detrimental impact to the body, diversely targeting a range of the human body systems. Common health conditions which are closely tied to metabolic imbalance include, non-alcoholic fatty liver disease, high blood pressure, low HDL cholesterol, high triglyceride levels, high fasting glucose levels, low fasting insulin levels, central obesity, polycystic ovarian syndrome, erectile dysfunction, glucose intolerance and mental fatigue (NIH, 2020). If an individual is concurrently suffering from a minimum of three or more of these health conditions, they are formally diagnosed with a pathology termed 'Metabolic Syndrome'. Metabolic Syndrome can increase the risk of a variety of secondary diseases, such as cardiovascular disease, stroke, and type 2 diabetes. Similar to the signs of metabolic

imbalance, the prevalence of Metabolic Syndrome is shockingly high. Across the United States, Metabolic Syndrome affects one out of every three people (CDC, 2020). Furthermore, as individuals age, the risk of Metabolic Syndrome dramatically increases, affecting nearly half of American adults over the age of 60 (CDC, 2020). Metabolic Syndrome is closely linked with other metabolic diseases such as obesity and diabetes. This is reinforced by the statistic that 85% of those suffering from Metabolic Syndrome are also diagnosed with a form of diabetes (CDC, 2020).

1.1 Influential Determinants of Metabolic Health

The causes of the previously mentioned ailments are complex and include a complicated network of frequently overlooked factors, such as genetic predisposition, socioeconomic status, and environmental influences. Though the influence of these factors on metabolic diseases remains unclear, research has found trends in genetic predispositions, socioeconomic status, and environmental influences that correlate with certain diseases. An influence on metabolism-related conditions that can, at times, be predictable is genetic predisposition, or heritability. Abdominal fat is one of the important risk factors that, when combined with others, result in Metabolic Syndrome. Heritability estimates for waist circumference range from 39–76% (Elder, 2009). Heritability estimates 8–51% for fasting insulin, 7–77% for fasting glucose, and 8–59% for insulin resistance assessed by the homeostatic model assessment of insulin resistance (HOMA-IR) – all measures are telling quantifications of metabolic health (Elder, 2009). For fasting HDL cholesterol, heritability estimates for 21–79% and 19–72% for fasting triglycerides, both risk factors for Metabolic Syndrome (Elder, 2009). These percentage estimates of

heritability mean that some individuals can only control for a small portion of their disposition to metabolic disease.

In addition to genetic predisposition, socioeconomic status has been shown to greatly impact metabolic health, more specifically CVDs (Schultz, 2018). Income level has been regularly associated with metabolic disease, this may be due to low-income patients experiencing disparities in standards of care because they lack the access to higher quality care (Schultz, 2018). Often linked with income level, education has been shown to inversely impact risk factors for CVD (Kucharska-Newton, 2011). Those with less education experience more metabolic risk factors, possibly attributed to the well-studied relationship between education and health literacy (Winkleby, 1992). Individuals with less education are often poorer in health literacy, these individuals tend to be less compliant with medication protocols, medical advice, and prevention strategies– these all contributing to all cause mortality (Bostock, 2012). Commonly used as a determinant of socioeconomic status, employment status has been shown to affect risk of metabolic diseases (Meneton, 2015). This may be due to increased levels of stress because of the potential job loss that led to the unemployment, the stress of being unemployed itself, or simply that individuals in poorer health are more likely to be unemployed than those with good health (Schultz, 2018).

We have seen that genetic predisposition and socioeconomic status influence health outcomes in very measurable ways. On top of these two influences, the environment in which an individual exists plays a prominent role in nearly every aspect of their health, especially in association to metabolic diseases. Environmental influences can encompass several elements, such as substance use, pollutants, residence location, safety, community

socialization, and many more. In a study done in young and healthy smokers versus non-smoking individuals, researchers saw that the risk of hypertriglyceridemia and low HDL levels were higher in smokers (Woo Kim, 2021). This same study determined that smokers had a 2.4-fold greater risk of developing Metabolic Syndrome than non-smokers (Woo Kim, 2021). Alcohol is another common substance that is well known to negatively impact health, excessive consumption has been linked to various components of Metabolic Syndrome, such as hypertension and obesity (Hernandez-Rubio, 2022). In a study comparing non-drinkers, light drinkers (mean: 5.1drinks/week), and heavy drinkers (mean: 30.7 drinks/week), heavy drinkers had the highest body mass index (BMI), waist circumference, obesity prevalence, hypertension, and hyperglycemia (Jung Choi, 2016). It was determined that heavy drinkers had a 1.71 times larger risk of developing Metabolic Syndrome than non-drinkers (Jung Choi, 2016). Substance use, such as smoking or drinking are usually voluntary behaviors and can be controlled for.

Harder to control for are environmental influences that individuals are typically born into with no choice: pollutants, neighborhood location and condition, and safety. Differences in neighborhood conditions impact the availability of resources, such as access to and promotion of healthy food, or an area conducive to physical activity. Overlooked features such as lowering the cost of healthy food or making exercise more accessible with easy walking paths, pleasant views, and access to resources via walking may affect metabolic health more than an educational campaign (Roux, 2003). A study done evaluating biological risk via cardiovascular, inflammatory, metabolic, and neuroendocrine health reported a relationship between poor health and neighborhood disadvantage (Barber, 2015). Evaluating BMI with neighborhood crime and safety in 673

adults in Chicago, Illinois, researchers found that over time, increasing individual- and neighborhood-level safety were linked with reduced BMI and subsequent decreased risk of cardiovascular health (Powell-Wiley, 2017). Environmental influences that, at times, cannot be controlled, similar to genetic predisposition and socioeconomic status, have tangible influence on metabolic health.

1.2 Glucose Homeostasis

Glucose homeostasis is an essential part of maintaining metabolic balance, as its disruption is implicated in several life-threatening pathologies, including Metabolic Syndrome. Glucose homeostasis is governed by two crucial peptide signals: insulin and glucagon. Insulin is synthesized and stored in the pancreas where it is released from pancreatic beta cells in response to a rise in blood glucose. A practical example of this occurs after eating a large meal. Insulin acts as a signal to stimulate the uptake of glucose by the cells as a response to high glucose levels in the blood. As a result, blood glucose levels return back down to a homeostatic range. Additionally, glucose uptake stimulated by insulin release enables the liver to create glycogen stores from glucose as a reserve in the case of an energy shortage.

The counterpart of glucose homeostasis via insulin involves another peptide known as Glucagon. Glucagon is stored and released from pancreatic alpha cells as the body senses a drop in blood glucose levels during a fasted or starved state. Glucagon acts as a signal to the liver and muscle cells, traveling through the bloodstream and binding to its G-protein-coupled-receptor (GPCR) known as the glucagon receptor. Once bound, Glucagon acts to break down glycogen stores into smaller glucose molecules, prompting its release into the blood. As a result, blood glucose levels rise and can return back to homeostatic levels.

Disruption of glucose homeostasis is linked to aging, a variable that makes nearly all metabolic conditions considerably worse (Spinelli, 2020). The risk factors produced by metabolic imbalance can be prevented or reduced with an improvement in metabolic balance. This improvement can be achieved through changes in diet and exercise.

1.3 Caloric Restriction and Glucose Homeostasis

One of the hallmark symptoms implicated in Metabolic Syndrome is an overall increase in blood glucose as a result of an impaired glucose tolerance and reduced insulin sensitivity (Mezhnina, 2020). In our lab, we study a diet that aims to address metabolic disorder, known as Caloric Restriction (CR). CR is a dietary paradigm in which the daily intake of food is reduced while avoiding malnutrition. CR is recognized for its benefits to health which drive the extension of lifespan in several mammals (Froy & Miskin, 2010; Mitchell et al., 2016). Mice placed on a long term CR diet have overall improved glucose homeostasis, meaning they have an improved ability to regulate their blood glucose levels. CR has been shown to positively impact metabolism through reduced blood glucose, improved glucose tolerance, and increased insulin sensitivity in CR mammals for over a hundred years (Mezhnina, 2020).

On a molecular level, CR affects several signaling pathways known to be associated with aging, cellular growth, and cellular metabolism such as the IGF, mTOR, AMPK, and sirtuin signaling pathways. (Balasubramanian, Howell, & Anderson, 2017). In the IGF-1 receptor pathway, a CR based eating regimen has been shown to reduce serum IGF-1 levels by up to 40%, potentially decreasing the risk of cancer and diabetes associated with the upregulation of IGF-1 and its action (Fontana et al., 2008)(Blackburn, 2017). CR diet has additionally been shown to downregulate the mTOR pathway through

decreased PI3K activation and increased AMPK phosphorylation (Arbor, 2018). These molecular changes ultimately act to reduce the rate of cell growth and metabolism, that of which is broadly implicated in the acceleration of cancer, diabetes, and aging. Thus, the controlled regulation of cell growth and metabolism through CR may be a beneficial method to combat these ailments. A known factor that plays a role in regulating these major metabolic and growth pathways is the circadian clock. The circadian clock generates 24-hour rhythms in physiology and metabolism in a diverse amount of organisms across taxa, working in most tissues and organs (Green et al., 2008) (Lowrey & Takahashi, 2004). For example, previous studies in our lab have shown the undisrupted circadian clock is necessary for a homeostatic modulation of mTOR signaling (Apelo, 2016). However, it has been shown that pharmacological inhibition of mTOR activity improves lifespan in both wild type mice and mice with a disrupted clock (Apelo, 2016). Notably, CR reduces mTOR signaling in wildtype mice, which is agreed upon to be one of the mechanisms by which CR extends lifespan. Interestingly, fluctuations in accessibility to food is a defining characteristic that contributes to physiological circadian rhythm, indicating an interaction between the circadian clock and eating patterns such as CR.

Mice represent the most commonly used mammalian model for research protocols due to their physiological, anatomical, and genetic resemblance to humans. For these similarities, the mouse is our model animal of choice. As a way to study and control for the contribution of circadian rhythm during a CR diet, mice used in our experiments are subjected to a 12 hour light-dark cycle (LD). The lights are on for 12 hours, then off for the next 12 hours. Mice are nocturnal animals, and they are at their most active during the

12 hour dark phase. Mice on the CR diet are given a 30% reduction in food and only fed once daily, two hours after the lights are turned off, as this makes physiological sense due to their nocturnality.

1.4 CR as a Periodic Fast

In addition to mice on the CR diet, there are mice on an Ad libitum (AL) diet. The mice on the AL diet have accessibility to food at all times during the LD. A food intake study previously done measuring food intake around the clock for both CR and AL diets shows that AL mice consume food at all times during the LD, but mainly consume the food during the dark phase (Velingkaar, 2020). The same food intake study revealed that CR mice eat their reduced food within two hours, leaving them in a state of fasting until they are fed again 22 hours later (Velingkaar, 2020). CR contains two components that are hypothesized to be beneficial: calorie restriction and fasting. Many have speculated that the benefits of CR are contributed to the fasting aspect of the diet. Because of this, diets that only implement the fasting aspect of CR without the calorie reduction have been studied.

One of the alternatives to CR that isolates the fasting variable is Time Restricted Feeding (TRF). TRF allows the subjects to feed freely, but only for a certain period of time. This introduces the aspect of periodic fasting which is similar to that of CR. TRF is also similar to intermittent fasting due to including an overall longer window of fasting (Jamshed, 2019). A pre-clinical trial study implementing TRF in humans placed one group on a 4 hour TRF, another on a 6 hour TRF, and the third group was without any food restriction (Cienfuegos, 2020). Both TRF groups demonstrated weight loss along with other mild benefits, but there is no definitive way to attribute these benefits to

fasting alone, as TRF does ultimately reduce total caloric intake in addition to the fast (Cienfuegos, 2020). A separate study examining TRF showed no benefits relevant to glucose metabolism after introducing humans to a time restriction of 8 hours to feed, without reducing calories (Lowe, 2020). Studies examining TRF have results inconsistent with one another, and this is possibly due to several variables at play, such as the time of TRF, the amount of time TRF is implemented, the caloric intake during these studies, or comorbidities in subjects (Lowe, 2020). Currently, the accumulated evidence does not suggest TRF to be an equal alternative both in efficacy and benefit to CR, but it is deemed as slightly more convenient in the context of practicality. Controlling a window of time in which a person eats is less complicated and more convenient than reducing caloric intake as a whole.

There is much to be investigated surrounding the mechanism of CR and whether fasting is the main contributor to these benefits. A study aimed to address the role of fasting in CR found that improvements in glucose homeostasis occur only in diets that incorporate fasting in tandem with reduced caloric intake (Velingkaar, 2020). In that study, 12 hour TRF with no caloric reduction was compared to AL and CR. An increase in insulin sensitivity and reduction in blood insulin was observed in both the TRF and CR diets compared to AL, but only CR had improvements in glucose homeostasis compared to AL (Velingkaar, 2020). Furthermore, they measured an important marker of glucose homeostasis, the glucose tolerance test (GTT). They found that CR mice had significantly improved glucose tolerance (100-200 mg/dL range for CR during the entire GTT) consistently compared to both the TR (100-300 mg/dL range for the entire GTT) and AL groups (100-400 mg/dL range for the entire GTT), while no difference was seen between

TRF and AL (Velingkaar, 2020). The researchers suspected that this difference in the effect on glucose homeostasis could be due to the difference in fasting durations between these groups, as the TRF mice were fasted for 12 hours and the CR mice were fasted for 22 hours (Velingkaar, 2020). An interesting question raised by Mezhnina et al. study is whether the duration of fasting contributes to glucose homeostasis, and if there is an optimal duration of fasting.

1.5 Glucose Tolerance

The GTT is an invaluable tool used to assess how fast glucose can be cleared in the blood and taken up by tissues. It provides a means of quantification for glucose homeostasis and regulation. Glucose tolerance is determined largely by insulin: the pancreas' ability to secrete it and tissue's response to it. As previously mentioned, insulin is released by the pancreas as it senses a rise in glucose in the blood. Insulin acts as a signal to cells/tissues to uptake the glucose from the blood, lowering blood glucose levels to a homeostatic range. Glucose tolerance is a measure of how well a subject's tissues can absorb the glucose, and this can be affected by several factors. A 'glucose tolerant' subject has blood glucose levels returning to a normal range relatively quickly after an influx of glucose. Broadly, a 'glucose intolerant' subject has a difficult time returning their blood glucose levels to a homeostatic range, requiring more time than a tolerant subject if they are capable. The actual glucose tolerance test involves an initial blood glucose measurement, followed by an administration of glucose, via injection, oral administration, etc. After the administration of glucose, blood glucose levels are measured at various times to examine how well the subject is able to clear the glucose from their blood, returning their blood glucose levels to a normal level.

Human applications of a GTT include those that detect type 2 diabetes, pre-diabetes, and gestational diabetes that develops during pregnancy.

Type 2 diabetes and pre-diabetes are very closely linked to insulin resistance, which coincides with glucose tolerance. Insulin resistance is developed when cells stop responding to the insulin signal to absorb glucose and blood glucose levels rise. The process begins when a large amount of glucose enters the bloodstream, in response the pancreas secretes more insulin to stimulate cells to uptake the glucose, and over time the cells stop responding to the large amount of insulin signaling making them insulin resistant (CDC, 2021). The pancreas senses all of the circulating glucose in the blood and it keeps secreting insulin in an attempt to make the cells respond. Insulin resistance is shown when the cells do not respond to the insulin, and blood sugar levels keep increasing (CDC, 2020). Remembering that glucose tolerance is dependent on the ability to clear glucose from the blood, there is a clear and well-known association between insulin resistance and glucose tolerance.

1.6 Insulin Signaling

Insulin plays a critical role in glucose homeostasis. Insulin resistance is caused by disruptions in the insulin signaling pathway. The insulin signaling pathway begins in the pancreas. The islets of Langerhans are a type of pancreatic cell that make up only 1-2% of the pancreas, aggregated together like small islands (myDr, 2018). The islets of Langerhans are endocrine alpha cells, which secrete glucagon and endocrine beta cells. These cells secrete insulin directly into the blood (myDr, 2018). Once insulin enters the bloodstream and binds to its tyrosine kinase receptor on insulin-response heavy tissues, such as the liver, adipose, and skeletal muscle, this binding induces phosphorylation on tyrosine residues on

insulin receptor substrate (IRS) proteins (Lizcano, 2002). PI3 kinase is recruited to the plasma membrane and phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2), converting PIP2 to phosphatidylinositol 4,5-triphosphate (PIP3) then recruiting protein kinase B (AKT) to the plasma membrane (Hooper, 2008). Pyruvate dehydrogenase kinase 1 (PDK1) then activates AKT and it enters the cytoplasm, where AKT phosphorylates and inactivates glycogen synthase kinase 3 (GSK3), promoting glucose storage as glycogen (Hooper, 2008). Insulin also signals AKT and PI3K to induce the translocation of glucose transporter, GLUT 4, to the plasma membrane to be the vehicle glucose uses to enter adipose and skeletal muscle cells (Hooper, 2008). The insulin signaling pathway in skeletal muscle and adipose tissue is demonstrated in **Figure 1.2**. In hepatocytes, GLUT2 is located at the plasma membrane; the glucose transporter does not require translocation (Leturque, 2005). The insulin signaling pathway in liver tissue is demonstrated in **Figure 1.2**. Upon insulin signaling in the liver, glucose is taken up by hepatocytes, which decreases gluconeogenesis and increases glycogen synthesis (LiverTox, 2018). Once glucose is inside the hepatocyte, it's converted into glucose-6-phosphate by hexokinase, trapping it inside the cell and allowing glycolysis to begin, making ATP (Diabetes in Control, 2012). In the liver, insulin signaling leads to the activation of glycogen synthase results in glycogen storages, as well as the inhibition of gluconeogenesis, the process by which the liver produces glucose, via inhibition of PEPCK and G6P (Diabetes in Control, 2012). The effects of insulin in the liver are more clearly shown in **Figure 1.2**.

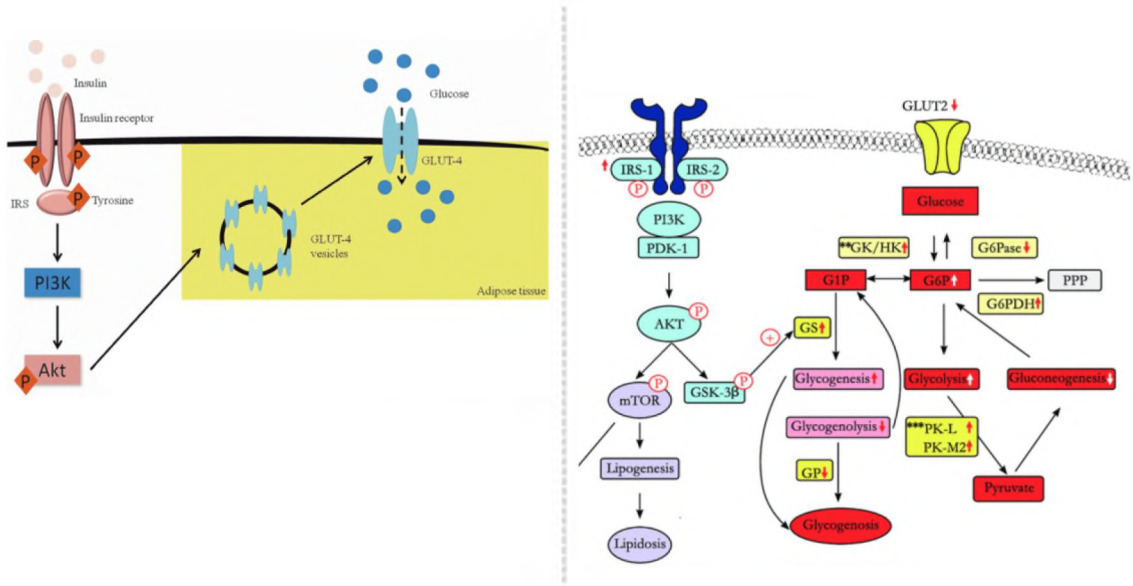

Figure 1.1 Insulin signaling pathway

On the left: Insulin signaling in skeletal muscle and white adipose tissue starts when insulin binds to its receptor. IRS 1/2 is phosphorylated in tyrosine, activating PI3K and Akt. Akt stimulates GLUT4 translocation to cell membranes thus allowing glucose to enter the cell. This figure was adapted from: https://www.researchgate.net/publication/262388226_Animal_Models_of_Nutritional_Induction_of_Type_2_Diabetes_Mellitus

On the right: Insulin signaling in hepatocytes of liver tissue starts when insulin binds to its receptor and the insulin signaling cascade results in phosphorylation/inactivation of glycogen synthase kinase-3beta (GSK-3β) and activation of glycogen synthase (GS). Glycogenolysis is reduced (GP, glycogen phosphorylase). Activation/phosphorylation of the Akt/mTOR complex induces de novo lipogenesis. Glucose is taken up by glucose transporter, GLUT2, phosphorylated to glucose-6-phosphate (G6P) by glucokinase and hexokinase (GK/HK) and metabolized in the glycolytic pathway to pyruvate or channeled via glucose-1-phosphate (G1P) into glycogen synthesis. Glucose-6-phosphate dehydrogenase (G6PDH) allows G6P into the pentose phosphate pathway (PPP). Gluconeogenesis is decreased. This figure was adapted from: https://www.researchgate.net/figure/Schematic-representation-of-the-molecular-mechanism-responsible-for-the_fig7_322132976

Insulin resistance is a hallmark sign of insulin signaling pathway disruption. The molecular mechanisms of insulin resistance include the downregulation of several parts of the insulin signaling pathway in skeletal muscle, adipose, and liver tissue. One mechanism of insulin resistance is the downregulation of the glucose transporter, GLUT4, in

adipocytes, leading to a cell's impaired ability to transport glucose from the blood into the cell (Sainai, 2010). Insulin resistance in skeletal muscle and adipocytes may also be due to downregulation of insulin receptor binding, phosphorylation of the tyrosine kinase receptor, and phosphorylation of IRSs, all of which negatively affect all downstream targets of the insulin signaling pathway (Invest, 2000). More tissue-specific examples include IRS downregulation in adipocytes of humans with type 2 diabetes, which reduces PI3K association with IRS and general activity, ultimately downregulating the signaling cascade that comes after (Invest, 2000). On the opposite end of that example, in skeletal muscle cells of humans with type 2 diabetes, IRS expression is normal, while PI3K activity associated with IRSs is reduced, again leading to the downregulation of the signaling cascade that follows (Invest, 2000). In rare cases, IRS mutations are possible, leading to insulin resistance due to impaired insulin signaling in adipose and skeletal muscle (Saini, 2010). Factors such as hyperinsulinemia, stress, mTOR, S6 kinase, inflammation, hyperlipidemia, obesity, hyperglycemia, and obesity may cause unusual serine phosphorylation of IRS proteins, lowering their ability to attract and interact with PI3K, in turn reducing all downstream effects of IRS (Saini, 2010). Mitochondrial dysfunction may also play a role in resistance, as it was found that in healthy elderly adults with acute skeletal muscle insulin resistance, there is approximately a 40% reduction in the rates of oxidative phosphorylation activity, connected to increased intramyocellular and intrahepatic lipid content (Petersen, 2003). Insulin resistance may be caused by several factors, individually or working in tandem, that directly affect glucose tolerance and overall glucose homeostasis.

1.7 Pancreatic Release of Insulin

Glucose tolerance is affected not only by insulin resistance, but it is also affected by pancreatic release of insulin. Before insulin resistance can even occur, the pancreas must secrete insulin. Pancreatic release of insulin behavior, whether it be downregulated or upregulated, directly affects glucose clearance from the blood by cells, which is glucose tolerance. To start, glucose stimulates the secretion of insulin by producing, then amplifying various signals in the insulin-secreting cells of the pancreas, beta cells (Henquin, 2000). Circulating blood glucose is uptaken by GLUT2, a facilitative glucose transporter in the plasma membrane of beta cells (Roder, 2016). Glucose then undergoes glycolysis in the beta cell, producing ATP, which gives an elevated ATP:ADP ratio (Roder, 2016). ATP-sensitive K+ channels close in response to the stimulus of the altered ratio, resulting in less K+ efflux from the cell, which depolarizes the membrane, opening voltage-gated Ca+ channels (Roder, 2016). A rise in intracellular calcium triggers the fusion of insulin-containing vesicles with the plasma membrane, mediated by proteins in the SNARE superfamily, resulting in insulin exocytosis and release of insulin in the blood (Roder, 2016). This insulin secretion process is known as glucose-triggered stimulus-secretion and several modulators have the potential to stimulate, amplify, or inhibit this process.

Prominent modulators of insulin release are G-protein-coupled receptors, glucose being a notable ligand for an amplification of insulin secretion in addition to its initial stimulus effect (Roder, 2016). G-protein-coupled receptors are followed by a signaling cascade that begins with the conversion of ATP (readily available due to glucose conversion to energy after a meal) into cyclic adenosine monophosphate (cAMP) by adenylate cyclase (Roder, 2016). cAMP activates protein kinase A (PKA), which

modulates K+ and calcium channels via phosphorylation, increasing the amount of calcium-sensitive insulin-containing vesicles that are readily releasable in pancreatic beta cells (Roder, 2016). In summary, glucose also increases the amount of insulin-containing vesicles that are readily releasable, amplifying the effect of insulin secretion. Free fatty acids (FFAs) also modulate insulin release, but in an inhibitory manner, through fatty acid metabolism. Short chain FFAs inhibit glucose-stimulated insulin secretion because of reduced glucose oxidation with the associated decreased ATP/ADP ratio (Roder, 2016). A study determined that short chain FFAs increased conversion of glucose to lactate (Ximenes, 2007). Short chain FFAs were seen to cause significant reductions in pyruvate decarboxylation (Ximenes, 2007). The researchers surmise that the decrease in glucose oxidation leads to a lower ratio of ATP:ADP (Ximenes, 2007). This lowered ratio downregulates the entire glucose-triggered insulin secretion process, which requires membrane depolarization caused by an elevated ATP:ADP ratio. The mentioned methods of insulin release modulation are only scratching the surface, there are several methods not mentioned, and even more still unknown.

Both insulin resistance and pancreatic insulin release must be examined to definitively determine the cause of dissimilar glucose tolerances and glucose homeostasis. Several studies have investigated if the benefits of CR are dependent on the fasting component of the diet. This study, too, focuses on the unanswered role of fasting as it relates to the glucose homeostasis benefits of CR by looking at an important measure of glucose homeostasis and regulation, glucose tolerance tests. We attempt to find out if fasting is responsible for the benefits of CR.

CHAPTER II

MATERIALS AND METHODS

2.1 Experimental Animals

All the animal studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC) of Cleveland State University (Protocol No. 21124-KON-S). The care and use of mice were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Cleveland State University. All animals were wild-type (WT) mice of C57BL/6J inbred strain background, housed under standard conditions of 12 hr light/dark cycle (LD 12:12) with lights turned on at 7:00 AM and turned off at 7:00 PM. All animals were fed a regular 18% protein chow diet except breeders which were maintained on a 19% protein diet (Harlan).

Feeding Regimen

The Ad-Libitum (AL) control group had unrestricted access to food across the day. Caloric restriction (CR) was started at 3 months of age for the CR experimental group of mice. For the first week of the diet, the animals were kept on 10% restriction, for the second week – on 20% restriction and on 30% restriction for the rest of their life and subsequent experimental measurements. The CR groups received their food once per day at ZT14 (two

hours after the light was turned off) as this is considered the most physiologically optimal time for food consumption, as mice are nocturnal animals and their activity peaks during the night. All groups had unrestricted access to water. Zeitgeber (external cue) is the light - hence the labeling "ZT" for every time point. The light was on at ZT0 (7:00AM) and off at ZT12 (7:00PM).

The experimental design is further described in **Figure 2-1**.

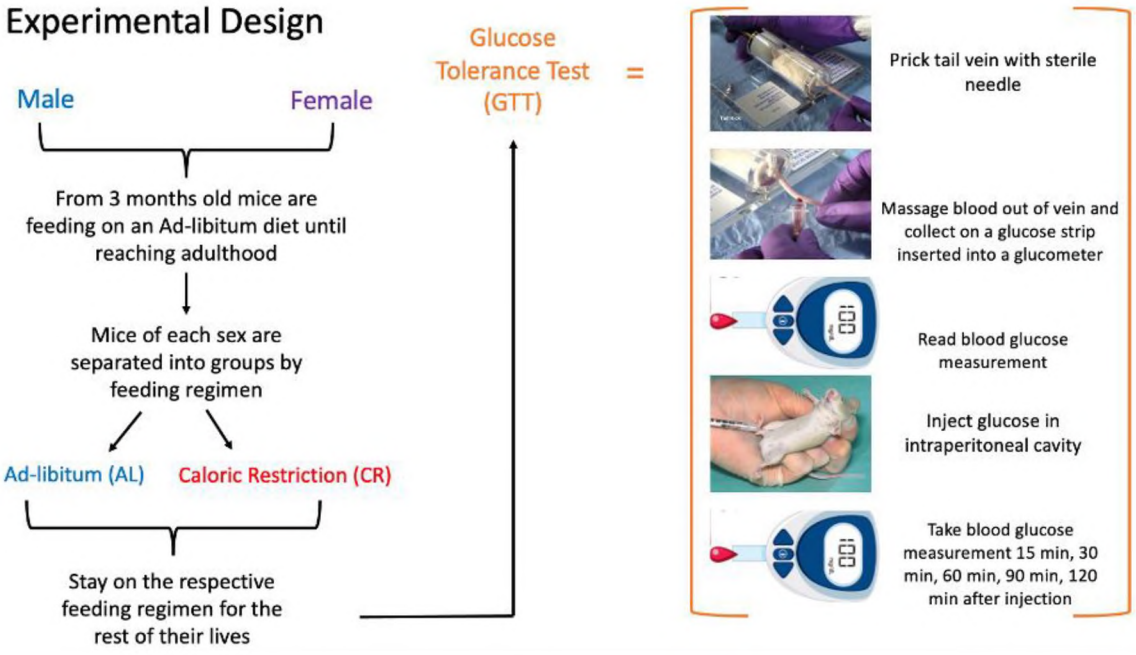

Figure 2-1. Experimental design

The flowchart represents the course of life of experimental mice up to the day of the glucose tolerance test. C57BL/6J wildtype mice on CR ages 4-6 months used for the glucose tolerance tests. From birth up to reaching adulthood (3-month-old) mice were fed on an ad-libitum diet (food present in the cage in unlimited amounts 24/7). After reaching 3 months of age, mice of each sex were split into groups by feeding regimen. CR was introduced to the respective group. CR mice received 70% of their daily food intake as a single meal once per day at ZT14, 2 hours after the light is turned off. Mice on all diets had unlimited access to water.

2.2 Blood Glucose Measurement

Blood for the analysis was collected through the tail vein, the nick was done using a sterile needle. Blood glucose was measured using CVS Advanced Health Blood Glucose Meter with CVS Health Advanced Glucose Meter Test Strips.

2.3 Intraperitoneal Glucose Tolerance Test (ip-GTT)

Initial blood glucose was measured using the method in chapter 2.2 before the test began. After initial measurement, we intraperitoneally injected glucose (1.2 g/kg body weight dose, Fisher, Hampton, NH) into the mouse and began a timer. Blood glucose was assayed, as detailed in chapter 2.2, in intervals as indicated: 15, 30, 60, 90, and 120 minutes after glucose injection. All parameters of the ip-GTTs were performed as indicated. Immediately following the test, mice were given food. Mice had access to water at all times.

2.4 Fasting

At the designated fasting time, mice that were to be fasted were taken from their cage and moved to a new, clean cage without food. The transfer to a clean cage at the start of the fast ensured the mice did not have crumbs to eat in their cage following food removal. All fasted mice had access to water at all times.

2.5 Statistical Analysis

Data are shown as average +/- standard error of the mean. Microsoft Excel and Matlab software packages were used for statistical analysis. To assay the effects of duration of fast and various diets - the analysis was performed using one and two-way repeated ANOVA, t-test, and Area Under the Curve (AUC). If the effect of diet or duration of fasting was found to be statistically significant, Bonferroni correction was used to calculate the p-

value for pairwise comparison. $P<0.05$ was considered as a statistically significant difference.

CHAPTER III

RESULTS

3.1 Effect of Diet on Blood Glucose Levels Throughout the Day

To begin to assess glucose homeostasis with the isolated variable of fasting from CR, blood glucose was measured at various time points throughout the day. CR mice, even having been fed at the start of the study, maintained the same blood glucose level throughout the 24 hour period. AL mice also showed a maintenance in blood glucose levels throughout the day. The fasted AL mice began the study with glucose measurements similar to AL, but finished the 24 hour fast with measurements similar to CR.

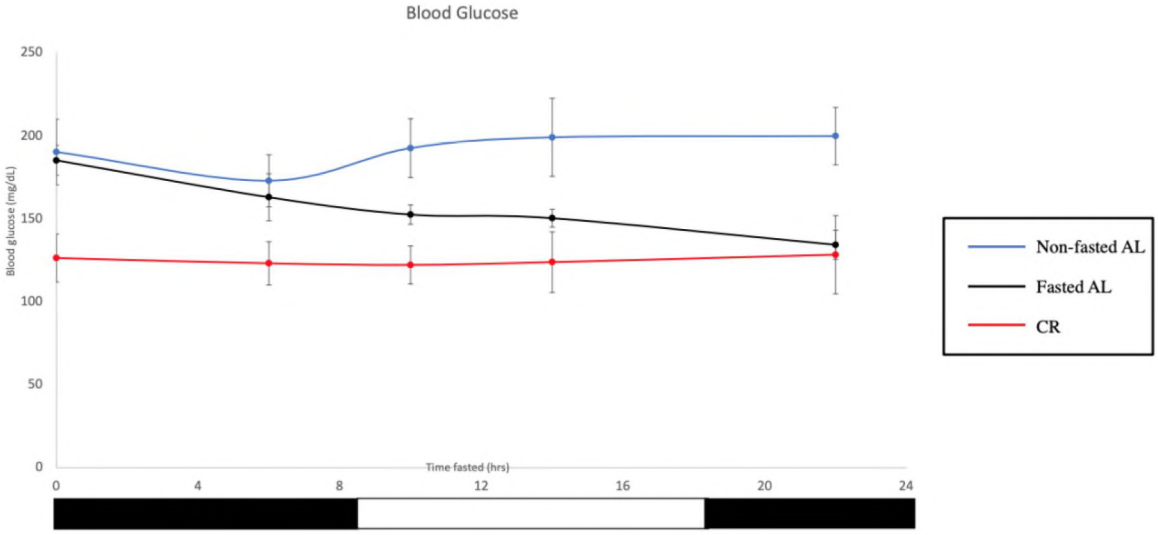

Figure 3-1. Effect of diet on blood glucose throughout the day

Blood glucose profiles throughout the day in mice (n >30 per diet) subjected to the following feeding regimens: ad libitum control (AL) – blue circles, solid line; 30% calorie restriction (CR) – red circles and solid lines; spontaneously fasted (SF) – black circles and solid lines. Data are represented as mean ± SEM. Light is on at ZT0 and off at ZT12. Food for the CR group was provided at ZT14 and food was taken away from the SF group at ZT14. The light bar on the bottom right represents 12 hours when the light was on. The dark bar – 12 hours when the light was off.

3.2 Effect of Fasted CR in Both Sexes on Glucose Tolerance

After confirming the advanced glucose regulation of CR mice from **Figure3-1** of measuring blood glucose levels throughout the day, the next step is further evaluating glucose regulation in CR with the aspect of fasting. The next approach used was a glucose tolerance test (GTT) to measure the body's ability to clear glucose from the blood after an influx of glucose from, in this case, an intraperitoneal injection. Three groups of CR mice were tested: a group of males following 10 hours of fasting, a group of females following 10 hours of fasting, and a group of males following 22 hours of fasting– the experimental setup is shown in **Figure 3-2**. GTT was also performed in non-fasted AL mice as a control. The results in **Figure 3-2a** show no significant difference between any of the CR groups as demonstrated by the Area Under the Curve graph in **Figure 3-2b**. Gender and duration of fast did not affect the glucose tolerance of the CR mice. All fasted CR groups showed significantly better glucose tolerance than the non-fasted AL control group. Area Under the Curve being much higher for the non-fasted AL control group in **Figure 3-2b** indicates a significant decrease in tolerance compared to all CR groups.

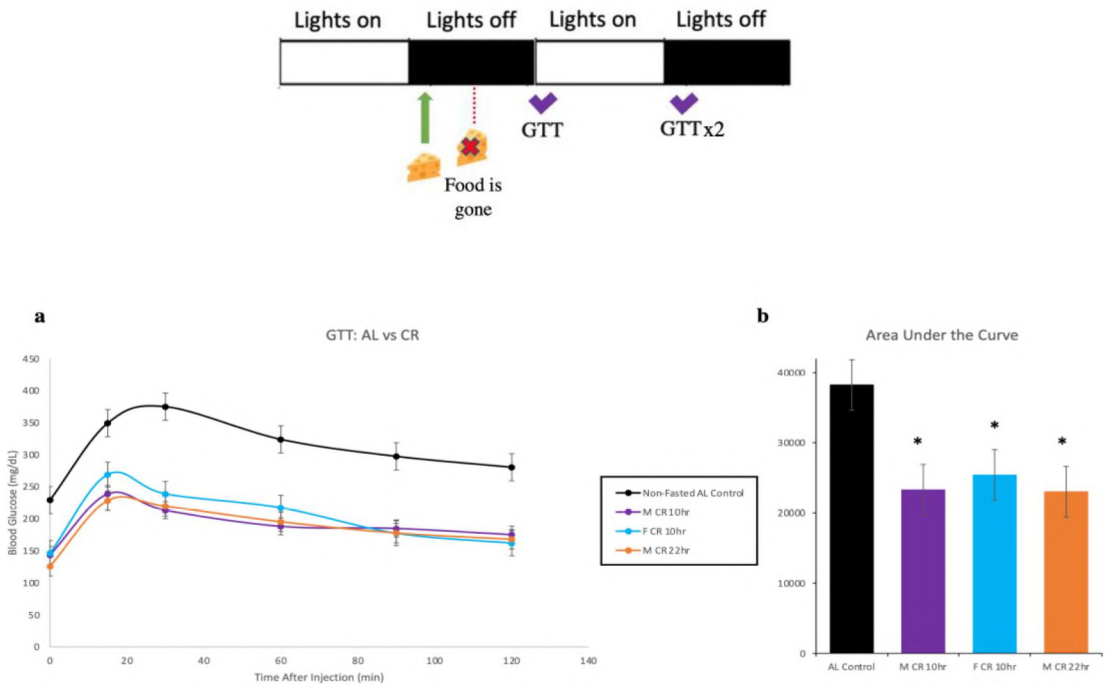

Figure 3-2. Glucose tolerance significantly improved upon male and female fasted CR mice compared to AL

Glucose tolerance test of mice on CR diet: black circles and solid lines - AL male mice (n=4); purple circles and solid lines – CR male mice following 10 hours of fasting (n=9), blue circles and solid lines – CR female mice following 10 hours of fasting (n=7); orange circles and solid lines – CR male mice following 22 hours of fasting (n=9). Statistically significant difference ($p<0.05$) between indicated groups at a particular time point. Data are represented as mean ± SEM. AL mice assayed at ZT14. Food for CR group was removed at ZT16 and assayed at ZT2 for 10 hour fast and ZT14 for 22 hour fast.

3.3 Fasting in Ad-libitum Mice Affects Glucose Tolerance

Fasted CR mice show advanced glucose compared to non-fasted AL mice, so we wanted to examine how fasting alone would impact glucose tolerance. In **Figure 3-1**, we saw that fasted AL mice showed improved glucose regulation compared to AL mice, but the regulation was not as advanced as mice on CR. To evaluate the effect of fasting alone on glucose tolerance, we fasted AL mice and performed GTT after completing the fast for comparison to non-fasted AL mice. Beginning with what we consider to be a short fast, regular AL mice had food removed from their cages spontaneously and were fasted

for 8 hours. The experimental setup is shown in **Figure 3-3**. As seen in **Figure 3-3a**, the 8 hours fasted AL mice show increased glucose tolerance compared to the non-fasted AL control group. The next step was to increase the length of fasting to 14 hours to assess the effect on tolerance, but there was no change in tolerance between the 14 hours fasted AL group and the non-fasted AL control group as represented in the similar AUC bars in **Figure 3-3b** and **Figure 3-3e**. Because there was no change in tolerance for 14 hours fasted mice, we believed that a possible plateau had been reached. To assess this, what we consider to be a long fast, 22 hours, was spontaneously applied to AL mice. The 22 hours fasted AL mice and the non-fasted AL control mice were assayed at the same time and a reduction in tolerance in the 22 hours fasted mice compared to the control was observed, seen in **Figure 3-3c**. In **Figure 3-3f** the AUC is increased in the 22 hours fasted group compared to the non-fasted AL group.

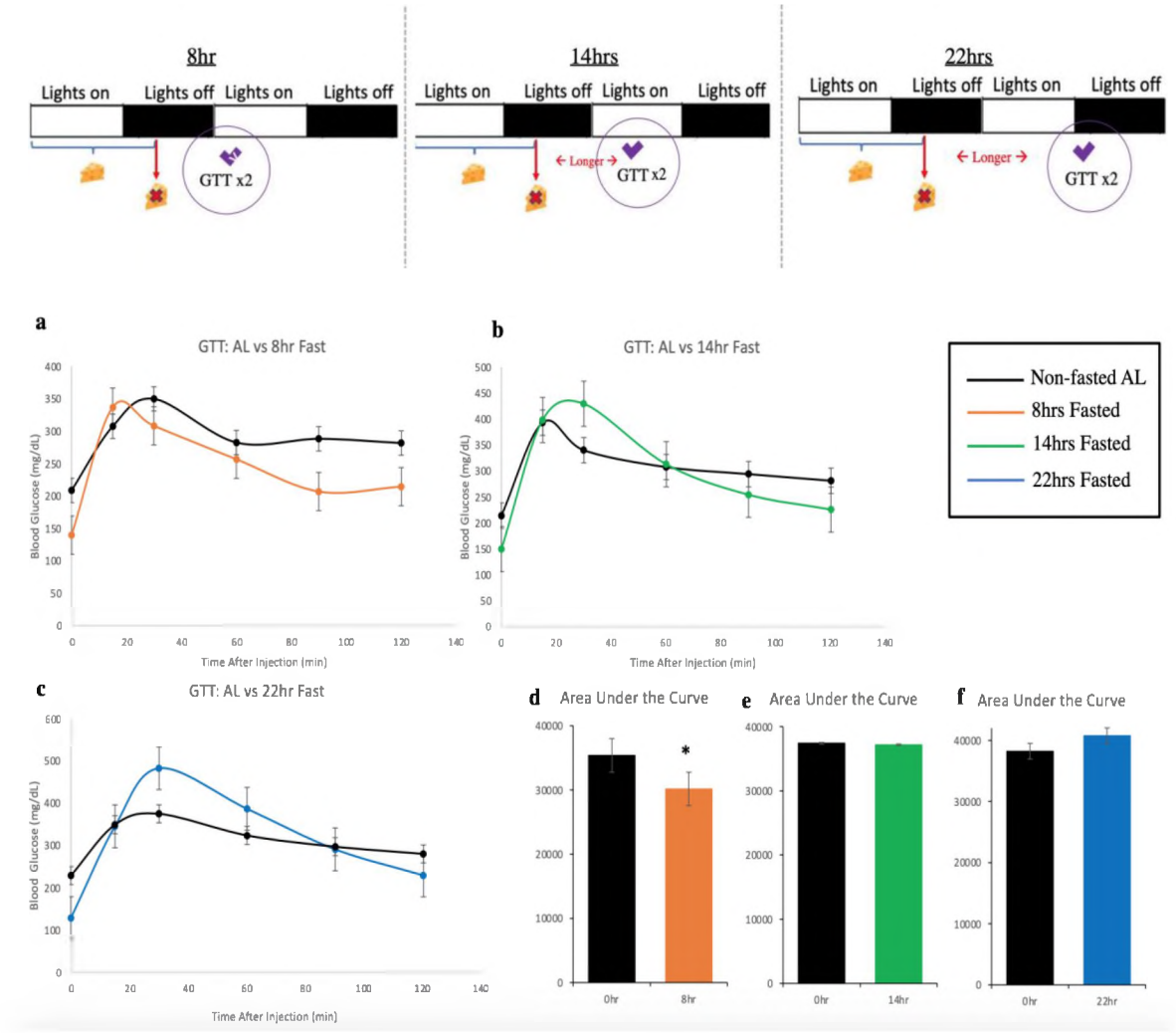

Figure 3-3. Different durations of fasting affect glucose tolerance in AL mice

A) Black circles and solid lines - AL male mice (n=4); orange circles and solid lines – AL male mice spontaneously fasted for 8 hours (n=4). Statistically significant difference ($p<0.05$) between indicated groups. Data are represented as mean ± SEM. Food for the fasted group was removed at ZT16 and GTT for both groups was performed at ZT0.
B) non-fasted AL mice and 14 hours fasted AL mice: black circles and solid lines - AL male mice (n=4); green circles and solid lines – AL male mice spontaneously fasted for 14 hours (n=4). No statistical significant difference ($p>0.05$) between indicated groups. Data are represented as mean ± SEM. Food for the fasted group was removed at ZT16 and GTT for both groups was performed at ZT6.
C) non-fasted AL mice and 22 hours fasted AL mice: black circles and solid lines - AL male mice (n=4); blue circles and solid lines – AL male mice spontaneously fasted for 22 hours (n=4). Statistically significant difference ($p<0.05$) between groups. Data are represented as mean ± SEM. Food for the fasted group was removed at ZT16 and GTT for both groups was performed at ZT14.

3.4 A Glucose Tolerance Comparison of Short Fasts to Long Fasts

Observable in **Figure 3-4a** is the opposite effect that short and long fasts have on glucose tolerance. This led to isolating a short and long fast to directly compare the two. In **Figure 3-4a** and **Figure 3-4c** are two different experimental setups to assay for differences in glucose tolerance due to fasting durations. The experimental setups are shown in **Figure 3-4**. In **Figure 3-4a**, fasting for GTT began at the same time and the actual GTT began at two different times, 8 hours after fasting began and 22 hours after fasting began. In this graph we can see that the 8 hours fasted group shows increased glucose tolerance compared to the 22 hours fasted group. This conclusion is also represented in **Figure 3-4b**, where the longer fasted group has a higher AUC value than the shorter fasted group. In **Figure 3-4c**, the AUC is representative of the 8 hour group having improved glucose tolerance with a lower AUC value. We did the experiment both ways to account for any time-of-day variance as another control. The results are consistent with the previous finding, observing that the 22 hours fasted group has decreased tolerance compared to the 8 hours fasted group. These findings are again represented by the AUC differences in **Figure 3-4d**, where the 22 hours fasted group has a higher AUC than the 8 hours fasted group.

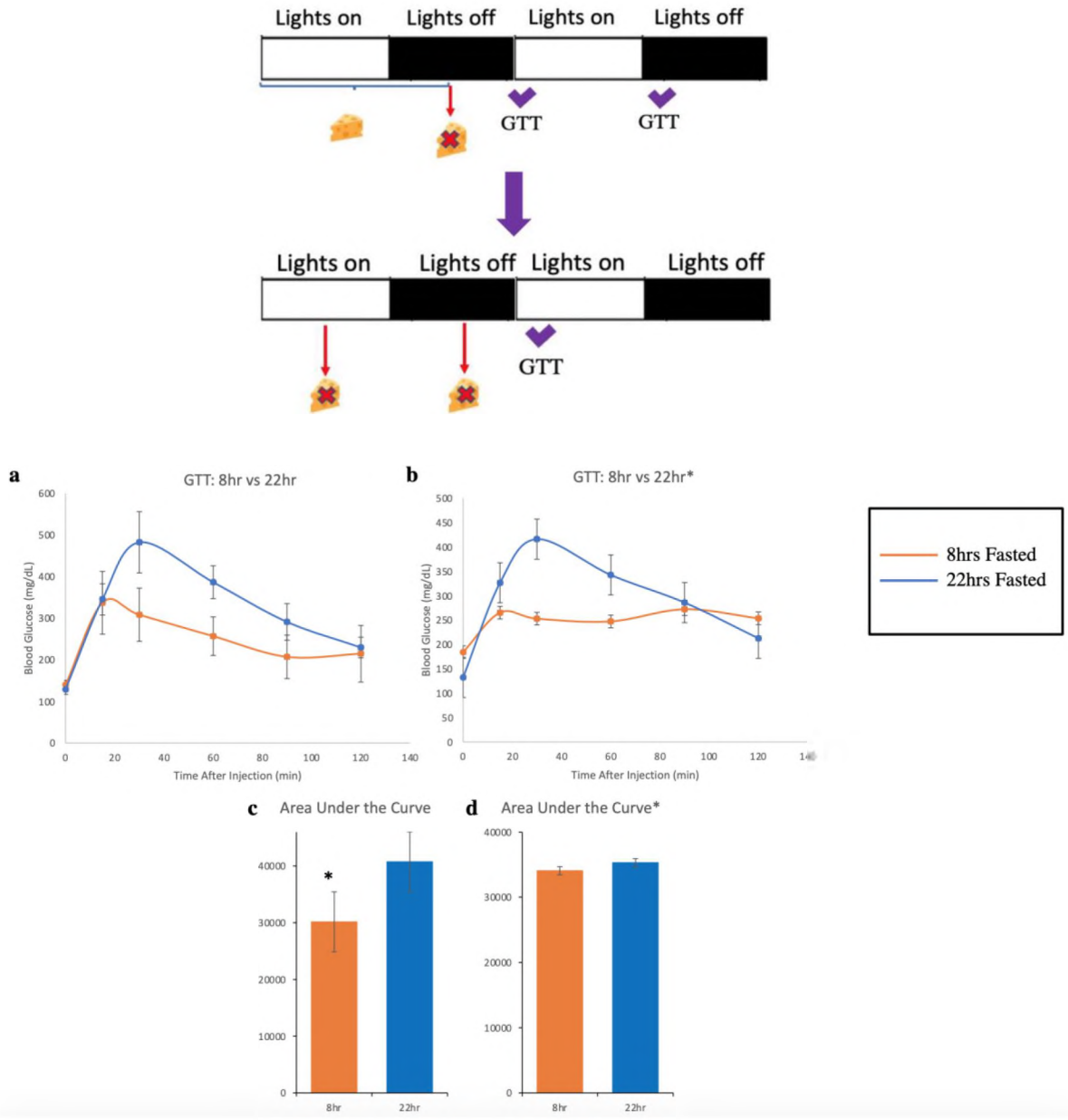

Figure 3-4. Increase in glucose tolerance in short fasts compared to long

Glucose tolerance test of 8 hours fasted AL mice and 22 hours fasted AL mice: orange circles and solid lines - AL male mice spontaneously fasted for 8 hours (n=4); blue circles and solid lines – AL male mice spontaneously fasted for 22 hours (n=4). Statistically significant difference ($p<0.05$) between groups. Data are represented as mean ± SEM. A/B)

Food for both groups was removed at ZT16 and GTT for 8 hours fasted mice performed at ZT0, 22 hours fasted mice at ZT14. C/D) Food for 8 hours fasted group, food was removed at ZT18 and at ZT4 for 22 hours fasted mice. Both GTTs were performed at ZT2.

3.5 Glucose Tolerance Test Around the Clock as a Control

There are variations in the experimental design of the GTTs, as seen in **Figure 3-2**, **Figure 3-3**, and **Figure 3-4**. In some circumstances, groups begin fasting at the same time and test at different times; in other circumstances, groups begin fasting at different times and test at the same time. This introduces another variable that could alter results of GTTs, so we introduced an experiment to control for this variable. GTTs were done to assess tolerance differences at different points around the clock, two done in the light phase and two done in the dark phase. Tests were performed on non-fasted AL mice at 4 different times in their 12 hour LD cycle, at two points in the dark phase and two points in the light phase. The experimental setup is shown in **Figure 3-5**. Glucose tolerance was not affected by the test being done at different times, seen in **Figure 3-5a**. **Figure 3-5b** shows the respective AUC values and confirms that there is no statistical difference between any of the tests, suggesting that time-of-day did not have an effect on tolerance. Duration of fasting was the most essential variable for differences in glucose tolerance.

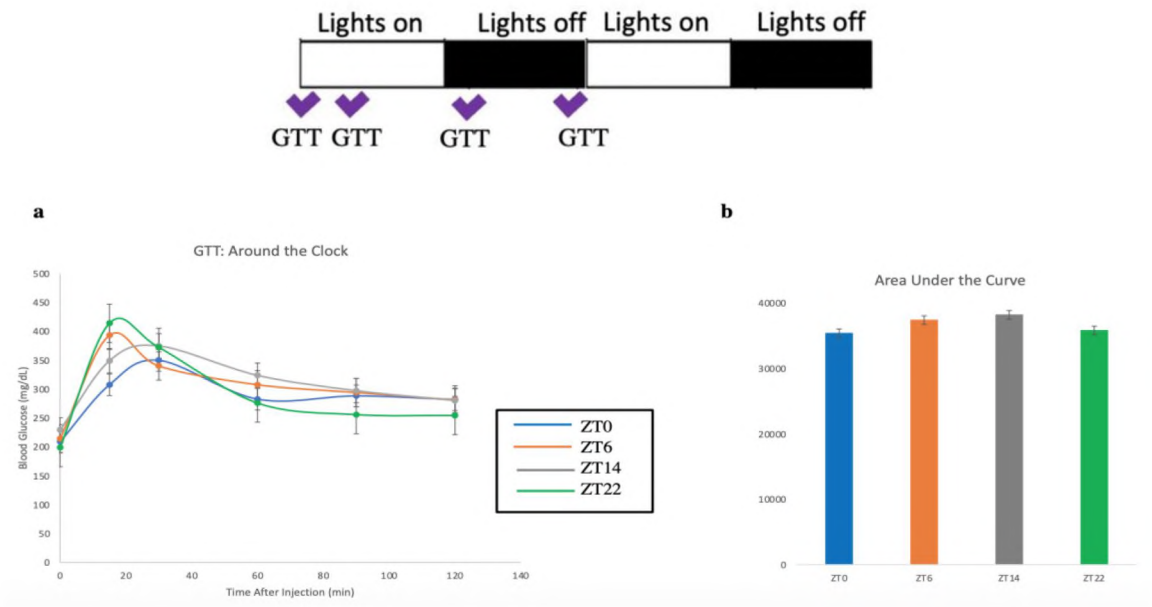

Figure 3-5. Glucose tolerance test around the clock as a control

Glucose tolerance test of non-fasted AL mice around the clock: blue circles and solid lines - AL male mice assayed at ZT0 (n=4); orange circles and solid lines – AL male mice assayed at ZT6 (n=4); gray circles and solid lines – AL male mice assayed at ZT14 (n=4); green circles and solid lines – AL male mice assayed at ZT22 (n=6). No statistical significance ($p>0.05$) between groups. Data are represented as mean ± SEM.

3.6 Glucose Tolerance in Long Fasts

Seeing a decrease in tolerance upon 22 hours of fasting was of particular interest to us. We decided to drastically increase the duration of fasting with this experiment, performing GTTs on mice fasted for 36 hours and 48 hours. The experimental setup is shown in **Figure 3-6**. With each increase in fasting duration, a significant decrease in tolerance was seen compared to non-fasted AL mice, shown in **Figure 3-6a**. This difference is confirmed by the correlating AUC chart in **Figure 3-6b**, the 48 hours fasted

group having the largest AUC, followed by the 36 hour group, then the non-fasted AL group.

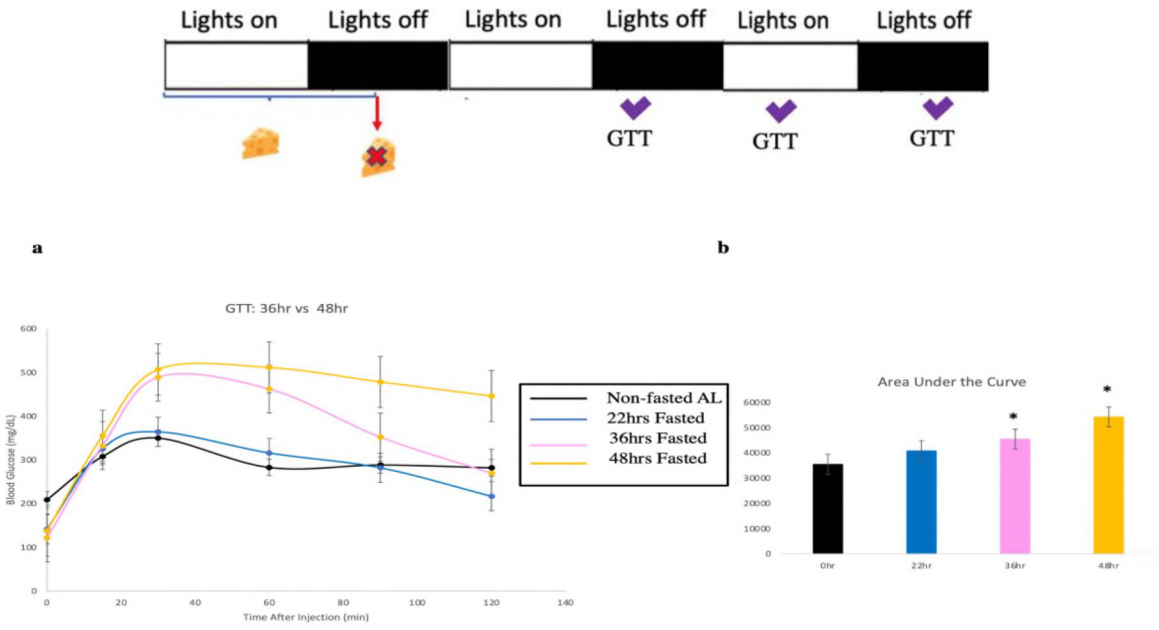

Figure 3-6. Glucose tolerance significantly decreased in 36 and 48 hour fasts

Glucose tolerance of 22 hours fasted AL mice, 36 hours fasted AL mice and 48 hours fasted AL mice: blue circles and solid lines - AL male mice fasted for 22 hours (n=4); pink circles and solid lines – AL male mice fasted for 36 hours (n=4); yellow circles and solid lines – AL mice fasted for 48 hours (n=4). Statistically significant difference ($p<0.05$) between groups. Data are represented as mean ± SEM. Food for all groups was removed at ZT16 and GTT was performed at ZT18 for 22 hours, ZT4 for 36 hours, and ZT16 for 48 hours.

CHAPTER IV

DISCUSSION

4.1 Glucose Tolerance in CR Mice

It has been established that CR mice have an advanced ability to maintain their glucose homeostasis. We are interested in evaluating the fasting component of CR, rather than the calorie reduction. GTT was performed following 10 hours of fasting in males and females on CR; and 22 hours of fasting in another group of males on CR, all compared to AL mice. Our observation reinforced what was currently known of CR. Regardless of gender and length of fast, CR mice have significantly improved glucose tolerance compared to AL mice. Glucose tolerance in both male and female groups were similar, with no statistical difference. It has been hypothesized that the beneficial longevity effects of CR are mediated by their increased insulin sensitivity/glucose tolerance. This notion has been questioned due to recent research discussing lifespan extension in CR mice while simultaneously observing a development of insulin resistance. It has been shown that mTORC2 (mammalian target of rapamycin complex 2) improves longevity in CR mice, but induces insulin resistance when disrupted (Yu, 2019). By adipose tissue-specific deletion of mTORC2 component, Rictor, researchers found that whole body insulin sensitivity decreased, but the same lifespan benefits typical

of CR mice were still observed (Yu, 2019). This leads us to believe that the benefits of CR relevant to glucose homeostasis may be independent of the lifespan benefits of CR. While there is discussion of this, wide acceptance would require extensive supportive evidence, as there is nearly a century of evidence supporting the relationship between CR's advanced glucose homeostasis and its longevity benefits.

The mechanism behind the improved glucose homeostasis and subsequent improved insulin sensitivity is not well established. It is postulated to be due to increased activation of certain molecules in the insulin signaling pathway (Li, 2017). Multiple studies have suggested that CR enhances insulin sensitivity through the insulin signaling pathway via increased activation of Akt, a downstream effector in the pathway (Li, 2017). In agreement with this suggestion, upregulation of insulin-stimulated PI3K (the upstream activator of AKT) signaling was shown to improve glucose tolerance (Schenk, 2011). It was also found that PI3K upregulation is necessary for the improvement in insulin sensitivity, but it is not upregulated by the typical PI3K activator, IRS, as IRS expression was the same in both CR and AL (Davidson, 2002). The researchers on this study surmise that this could be due to CR 1) acting on a post-PI3K event in the insulin signaling pathway; 2) altering the localization of components in the insulin signaling pathway; 3) working with a separate, unknown pathway acting in tandem with PI3K (Davidson, 2002). It is widely accepted that whichever component of the insulin signaling pathway is upregulated in CR, the improved insulin-stimulated glucose transport can be attributed to a pronounced translocation of the GLUT-4 glucose transporter to the plasma membrane of muscle and adipose compared to AL mice.

While fasting is a component of CR, fasted mice and CR mice are considered to be on two different diets that yield different results. It is conflicting to state that the 10 hour fast and 22 hour fast performed on CR mice has the same effect that occurred with AL mice. Fasted AL mice in all experiments in this study were placed on a spontaneous fast from the AL diet, which is different from the fasting occuring in CR mice. As mentioned, our lab found that CR mice are technically fasted for 22 hours a day, feeding for only 2 hours (Velingkaar, 2020). The GTT that was termed a 10 hour fast, was performed 10 hours after the mice were finished feeding, so no spontaneous fast was implemented. The same design was adopted for the 22 hour fast GTT. These mice were assayed 22 hours after feeding, right before their next designated feed at ZT14. To be precise, the CR mice were not placed on a forced, spontaneous fast. The assayed CR mice were simply tested within the normal routine of their daily cycle. This limitation can be addressed by extending the fast past their daily fast of 22 hours and performing the GTT to examine if, in a spontaneously fasted state, their glucose tolerance is still improved as we observed in this study.

The mechanism by which CR improves glucose metabolism and prevents or reduces insulin resistance remains largely unknown, but is thought to be attributed to a network of factors. Considering the two systems that control glucose tolerance: pancreatic release of insulin and the insulin signaling pathway in tissue; the common hypothesis is that CR acts on the insulin signaling pathway to effect its improvements in glucose homeostasis.

4.2 Changes in Glucose Tolerance in Response to a Short Fast

We observed that glucose tolerance improved in AL mice fasted for 8 hours compared to non-fasted AL mice. For the purpose of our study, 8 hours is considered a short fast. Short fasts in hopes of improving metabolic health have been gaining significant popularity in recent years with the rise of intermittent fasting (IF). IF involves a voluntary fast, where an individual eats freely within a restricted window of time, and fasts for the remaining hours in the day. The draw to IF being that it forces the body to first use fat stores as energy to produce weight loss, instead of glucose from frequent meals throughout the day. The link between IF and glucose homeostasis is of recent relevance because of the rising popularity of the diet. Studies have shown that IF is linked to increased glucose tolerance and insulin sensitivity (Trumpfeller, 2020). Some physicians even suggest forms of IF to diabetic patients to mitigate symptoms of the disease (Albosta, 2021). However, as discussed in **chapter 1.3**, it is challenging to determine whether the increase in insulin sensitivity is due to the fasting or inevitable long-term calorie reduction involved in most fasting diets similar to IF.

The effect of calories should play a less significant role in the improved glucose tolerance observed. Regardless of the differences between the spontaneous short fast and fasting diets that include reduction of calories, our results are consistent with literature. Short fasts were observed to improve glucose tolerance, but the mechanism through which it does may be dissimilar to those that reduce calories, and remains largely unknown.

Glucose tolerance is ultimately dependent on insulin. Insulin stimulates the uptake of glucose into tissue, clearing it from blood and returning blood glucose levels back to a

homeostatic range. Because we observed differences in glucose tolerance in fasted AL mice and non-fasted AL, we must investigate insulin– its release from the pancreas and subsequent signaling pathway in tissue. While still unclear, it is widely thought that improvements in glucose homeostasis from fasting originates from alterations in the insulin signaling pathway, not alterations in the pancreas. A study evaluating insulin signaling and glucose metabolism in lean versus obese adults reported an increase in insulin-stimulated Akt phosphorylation after a 12 hour fast in lean adults compared to obese adults (Bergman, 2007). Another study showed IRS2 associated PI3K activity increased in the liver of fasted mice (Kubota, 2008). IRS2 is one of the first proteins involved in insulin signaling as it activates PI3K. This study's result would then suggest that fasting increases IRS2 activation of PI3K, resulting in increased insulin sensitivity and improved glucose tolerance.

It would be useful to assess at what point in fasting is improved glucose tolerance induced. We could potentially see a more significant improvement in glucose tolerance upon a shorter fast of 4-6 hours. It would also be useful to extend the short fast to 10 or 12 hours to assess at what point glucose intolerance begins to develop, as we saw no change between AL and the mice fasted for 14 hours. It is important to note that basal metabolic rate, an efficacy measure of metabolism, is nearly seven times higher in mice than in humans (Agoston, 2017). This difference in metabolic rates makes it difficult to translate findings from mice to humans. For example, the improvement upon short fasting observed in this study does not directly imply that an 8 hour fast in humans produces the same result we observed in mice. This consideration should be carefully reviewed when discussing any translation of findings from mice to humans. It is in our interest to assay

various proteins involved in the insulin signaling pathway upon short fasting in AL mice and compare these results to non-fasted AL mice. Findings may help discover a potential target in the insulin signaling pathway that can be used as a form of therapy for metabolic disease.

4.3 Changes in Glucose Tolerance in Response to a Long Fast

When we performed the GTT for the 22 hour fast in AL mice and compared it with non-fasted AL mice, we observed a decrease in glucose tolerance. This was of particular interest because we were not anticipating a decrease in tolerance. We saw an improvement in tolerance upon an 8 hour fast and relatively no change in tolerance upon a 14 hour fast. The blood glucose measurements during the GTT were significantly increased compared to non-fasted AL mice and the 8 hour fasted AL mice. Because of the unusually high blood glucose measurements, we postulated that there must be a point at which a plateau is reached. The increase of fasting duration from 22 hours to 36 hours decreased tolerance even more, and blood glucose reached very high levels, peaking at ~500 mg/dL at 30 minutes. The data suggested that the longer the fast was forced, the bigger the decrease in glucose tolerance.

We decided to implement an even longer fast to further confirm the observed trend. AL mice spontaneously fasted for 48 hours demonstrated a GTT that resembles a plateau, with extreme glucose intolerance. The peaks at 30 minutes after injection for both the 36 hour fasted mice and the 48 hour fasted mice are similar, both much higher than the 22 hour fasted mice. However, the 48 hour fasted mice could not bring their blood glucose levels back down in the same time that the 36 hour fasted mice could. There is a clear trend showing that spontaneous long fasts in AL mice decrease glucose

tolerance compared to non-fasted AL mice. Within this trend, we showed that the longer the fast, the more intolerant the mice become.

Our results support the current literature that has evaluated glucose homeostasis in relation to different lengths of fasting in various models. One study evaluated insulin signaling in lean and obese adults fasted for either 12 hours or 48 hours (Bergman, 2007). The researchers found a decline in insulin signaling in both lean and obese adults upon a 48 hour fast compared to lean and obese adults upon a 12 hour fast (Bergman, 2007). The decline in insulin signaling is a development of insulin resistance, a possible explanation for the development of glucose intolerance. Just as we speculated about what is responsible for improved glucose tolerance upon short fasting, we must investigate the same question in long fasts. The question then is whether pancreatic insulin release or insulin signaling is responsible for the decrease in glucose tolerance?

We cannot definitively state that this is due to insulin resistance, because the mechanism responsible for tolerance differences may be altered before insulin is even secreted. It is possible that pancreatic insulin release could be reduced upon long lengths of fasting, or that the insulin signaling pathway is disrupted causing insulin resistance. In **chapter 1.6** we discussed the inhibitory effects of FFA on insulin secretion and it has been found that FFA concentration increases nearly eightfold during fasting (Salgin, 2012). There is a general agreement that this increase in FFA downregulates insulin action by inhibiting pancreatic insulin secretion (Bergman, 2007). There is also evidence that the increased FFA concentration acts on the insulin signaling pathway by reducing IRS associated PI3K activity or reducing IRS phosphorylation activation. Both of these IRS associated reductions would decrease glucose transport via GLUT4 in skeletal

muscle (Griffin, 1999). A study also found that mTOR signaling was significantly down in skeletal muscle tissue of adult males following 72 hours of fasting (Vendelbo, 2014). As a reminder, mTOR signaling is one of the mechanisms by which CR mice enact their advanced glucose homeostasis. Downregulation of mTOR signaling in long fasts is consistent with the decrease in glucose tolerance we observed in long fasts.

It is less common to observe glucose tolerance being affected by pancreatic insulin release behavior, rather than insulin signaling, but it has been observed. For example, we discussed the inhibitory effect FFAs have on pancreatic insulin secretion. It was also found that higher pancreatic fat content in humans was associated with higher BMI, waist circumference, and impaired glucose tolerance compared to those with lower pancreatic fat content (Heni, 2010). It was determined that pancreatic fat had a negative impact on insulin secretion and may be a factor in pancreatic beta cell dysfunction resulting in impaired glucose homeostasis (Heni, 2010). However, another study showed pancreatic fat content was correlated with higher fasting glucose levels and impaired glucose tolerance without a direct correlation to disruption in beta cell function (Zijl, 2011). While this study did not find a direct correlation between impaired glucose tolerance and beta cell dysfunction, the idea that beta cell dysfunction is responsible for impairment of pancreatic insulin release is widely accepted. Decline in beta cell function is well known to occur in the time before diagnosis of type 2 diabetes and is central in the disease's progression (Halban, 2014). Addressing beta cell dysfunction is of high importance in the development of therapeutics for type 2 diabetes and could potentially be beneficial for those suffering from similar metabolic disorders.

Our findings were consistent with current literature in that prolonged fasting does impair glucose homeostasis. The next step is determining the cause of the intolerance development. Assaying for insulin after a glucose administration will be useful for determining if intolerance is due to disruptions in pancreatic insulin release. With the same mindset moving forward discussed in **chapter 4.2**, we would like to assay various proteins involved in the insulin signaling pathway upon long fasts in AL mice and compare results with non-fasted AL mice. Findings of this, too, may help discover a potential target in the insulin signaling pathway that can be used as a form of therapy for metabolic disease.

4.4 Future Directions

In our future studies, we plan on assaying for insulin release in fasted (various lengths), non-fasted, and CR mice after a glucose injection. This requires collecting blood before the injection and various time points afterward. The blood collection would be spun, and serum extracted with an ELISA to follow in order to examine insulin levels. This would help uncover if the differences in glucose tolerance are due to insulin release behavior. Another future plan is to assay for insulin signaling molecules to assess if tolerance differences are due to tissue insensitivity/resistance to insulin. This would be done with an insulin tolerance test, similar to a GTT, or a tissue collection-based method. This would entail injecting insulin into mice, then collecting liver and skeletal muscle tissue. A Western blot would follow collection to detect possible changes in the insulin signaling pathway.

CHAPTER V

CONCLUSION

It is widely known that mice on a CR diet experience several benefits, most relevant to this study being the advanced glucose homeostasis and regulation. This was confirmed by the steady across-the-day blood glucose measurement and the several GTTs done on CR mice showing advanced glucose tolerance compared to mice of different diets. CR mice are able to maintain a low and steady blood glucose level at all times, even after feeding, and can bring their blood glucose level back down to homeostatic range after a glucose injection. Being that there are two aspects of the CR diet, fasting between meals and the 30% food reduction, we set out to investigate the role of fasting in the benefits of CR. Ultimately, we found that fasting does affect glucose tolerance, but this effect is very different depending on the duration of the fast. It was observed that short fasts increase glucose tolerance compared to non-fasted AL mice, while long fasts decrease glucose tolerance. When comparing GTTs of all fasting durations with the CR groups, we saw that the CR groups still had significantly better glucose tolerance than any of the fasted AL groups. This leads us to believe that fasting is not the only contributor to the improved glucose tolerance and regulation of the CR mice.

Figuring out why fasting is not the main contributor to the advanced glucose tolerance of CR begins with identifying what determines glucose tolerance in the first place, which is tissue response to insulin. As further discussed in the previous chapter, the tissue response to insulin could be affected by several things, including pancreatic insulin release behavior or insulin resistance/insulin signaling behavior in the tissue itself. Although fasting does have a role in affecting glucose tolerance in CR mice, another unknown factor plays a more important role.

REFERENCES

Agoston, D. v. (2017). How to Translate Time? The Temporal Aspect of Human and Rodent Biology. *Frontiers in Neurology*, *8*(MAR), 92. https://doi.org/10.3389/FNEUR.2017.00092

Albosta, M., & Bakke, J. (2021). Intermittent fasting: is there a role in the treatment of diabetes? A review of the literature and guide for primary care physicians. *Clinical Diabetes and Endocrinology 2021 7:1*, *7*(1), 1–12. https://doi.org/10.1186/S40842-020-00116-1

An overview of insulin signaling pathways | Abcam. (2008). https://www.abcam.com/pathways/overview-of-insulin-signaling-pathways

Anderson, R. M., & Weindruch, R. (2010). Metabolic reprogramming, caloric restriction and aging. *Trends in Endocrinology and Metabolism: TEM*, *21*(3), 134. https://doi.org/10.1016/J.TEM.2009.11.005

Arbor, S. (2018). Where and How in the mTOR Pathway Inhibitors Fight Aging: Rapamycin, Resveratrol, and Metformin. *Resveratrol - Adding Life to Years, Not Adding Years to Life*. https://doi.org/10.5772/INTECHOPEN.79338

Arriola Apelo, S. I., & Lamming, D. W. (2016). Rapamycin: An InhibiTOR of Aging Emerges From the Soil of Easter Island. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *71*(7), 841. https://doi.org/10.1093/GERONA/GLW090

Balasubramanian, P., Howell, P. R., & Anderson, R. M. (2017). Aging and Caloric Restriction Research: A Biological Perspective With Translational Potential. *EBioMedicine*, *21*, 37–44. https://doi.org/10.1016/J.EBIOM.2017.06.015

Barber, S., Hickson, D. A., Kawachi, I., Subramanian, S. v., & Earls, F. (2016). Neighborhood Disadvantage and Cumulative Biological Risk Among a Socioeconomically Diverse Sample of African American Adults: An Examination in the Jackson Heart Study. *Journal of Racial and Ethnic Health Disparities*, *3*(3), 444–456. https://doi.org/10.1007/S40615-015-0157-0

Bergman, B. C., Cornier, M. A., Horton, T. J., & Bessesen, D. H. (2007). Effects of fasting on insulin action and glucose kinetics in lean and obese men and women. *American Journal of Physiology - Endocrinology and Metabolism*, *293*(4), 1103–1111. https://doi.org/10.1152/AJPENDO.00613.2006/ASSET/IMAGES/LARGE/ZH10110750960003.JPEG

Bostock, S., & Steptoe, A. (2012). Association between low functional health literacy and mortality in older adults: longitudinal cohort study. *BMJ (Clinical Research Ed.)*, *344*(7852). https://doi.org/10.1136/BMJ.E1602

Choi, S. J., Lee, S. I., & Joo, E. Y. (2016). Habitual Alcohol Consumption and Metabolic Syndrome in Patients with Sleep Disordered Breathing. *PLOS ONE*, *11*(8), e0161276. https://doi.org/10.1371/JOURNAL.PONE.0161276

Cienfuegos, S., Gabel, K., Kalam, F., Ezpeleta, M., Wiseman, E., Pavlou, V., Lin, S., Oliveira, M. L., & Varady, K. A. (2020). Effects of 4- and 6-h Time-Restricted Feeding on Weight and Cardiometabolic Health: A Randomized Controlled Trial in Adults with Obesity. *Cell Metabolism*, *32*(3), 366-378.e3. https://doi.org/10.1016/J.CMET.2020.06.018/ATTACHMENT/B2FDE0A0-8674-4FD8-816D-63B818542220/MMC1.PDF

Davidson, R. T., Arias, E. B., & Cartee, G. D. (2002). Calorie restriction increases muscle insulin action but not IRS-1-, IRS-2-, or phosphotyrosine-PI 3-kinase. *American Journal of Physiology - Endocrinology and Metabolism*, *282*(2 45-2), 270–276. https://doi.org/10.1152/AJPENDO.00232.2001/ASSET/IMAGES/LARGE/H10220680005.JPEG

Deyang Yu, J. L. T., Shany E. Yang, ..., Jacqueline A. Brinkman, F. A. S., & Dudley W. Lamming. (2019). Calorie-Restriction-Induced Insulin Sensitivity Is Mediated by Adipose mTORC2 and Not Required for Lifespan Extension. *Cell Reports*, *29*, 236–248. https://www.cell.com/cell-reports/pdf/S2211-1247(19)31146-5.pdf

Diabetes and cancer: What you should know | MD Anderson Cancer Center. (n.d.). Retrieved April 20, 2022, from https://www.mdanderson.org/publications/focused-on-health/Diabetes-and-cancer.h26Z1591413.html

Fontana, L., Weiss, E. P., Villareal, D. T., Klein, S., & Holloszy, J. O. (2008). Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. *Aging Cell*, *7*(5), 681. https://doi.org/10.1111/J.1474-9726.2008.00417.X

Froy, O., & Miskin, R. (2010). Effect of feeding regimens on circadian rhythms: implications for aging and longevity. *Aging*, *2*(1), 7–27. https://doi.org/10.18632/AGING.100116

Green, C. B., Takahashi, J. S., & Bass, J. (2008). The meter of metabolism. *Cell*, *134*(5), 728–742. https://doi.org/10.1016/J.CELL.2008.08.022

Griffin, M. E., Marcucci, M. J., Cline, G. W., Bell, K., Barucci, N., Lee, D., Goodyear, L. J., Kraegen, E. W., White, M. F., & Shulman, G. I. (1999). Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes*, *48*(6), 1270–1274. https://doi.org/10.2337/DIABETES.48.6.1270

Halban, P. A., Polonsky, K. S., Bowden, D. W., Hawkins, M. A., Ling, C., Mather, K. J., Powers, A. C., Rhodes, C. J., Sussel, L., & Weir, G. C. (2014). β-Cell Failure in Type 2 Diabetes: Postulated Mechanisms and Prospects for Prevention and Treatment. *Diabetes Care*, *37*(6), 1751. https://doi.org/10.2337/DC14-0396

Heni, M., Machann, J., Staiger, H., Schwenzer, N. F., Peter, A., Schick, F., Claussen, C. D., Stefan, N., Häring, H. U., & Fritsche, A. (2010). Pancreatic fat is negatively associated with insulin secretion in individuals with impaired fasting glucose and/or impaired glucose tolerance: a nuclear magnetic resonance study. *Diabetes/Metabolism Research and Reviews*, *26*(3), 200–205. https://doi.org/10.1002/DMRR.1073

Henquin, J. C. (2000). Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes*, *49*(11), 1751–1760. https://doi.org/10.2337/DIABETES.49.11.1751

Hernández-Rubio, A., Sanvisens, A., Bolao, F., Cachón-Suárez, I., Garcia-Martín, C., Short, A., Bataller, R., & Muga, R. (2022). Prevalence and associations of metabolic syndrome in patients with alcohol use disorder. *Scientific Reports 2022 12:1*, *12*(1), 1–7. https://doi.org/10.1038/s41598-022-06010-3

Holm Vendelbo, M., Buch Møller, A., Christensen, B., Nellemann, B., Frederik Forrest Clasen, B., Sreekumaran Nair, K., Otto Lunde Jørgensen, J., Jessen, N., Møller, N., & Trajkovic, V. (2014). *Fasting Increases Human Skeletal Muscle Net Phenylalanine Release and This Is Associated with Decreased mTOR Signaling*. https://doi.org/10.1371/journal.pone.0102031

How the Liver Affects Insulin and Vice Versa: Part 1 Carbohydrate Metabolism. (n.d.). Retrieved April 25, 2022, from https://www.diabetesincontrol.com/how-the-liver-affects-insulin-and-vice-versa-part-1-carbohydrate-metabolism/

Insulin. (2018). *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*. https://www.ncbi.nlm.nih.gov/books/NBK548016/

Insulin Resistance and Diabetes | CDC. (n.d.). Retrieved April 18, 2022, from https://www.cdc.gov/diabetes/basics/insulin-resistance.html

Intermittent Fasting and Insulin Resistance: How the Two Are Connected. (n.d.). Retrieved April 20, 2022, from https://simple.life/blog/intermittent-fasting-and-insulin/

Kahn, B. B., & Flier, J. S. (2000). Obesity and insulin resistance. *Journal of Clinical Investigation*, *106*(4), 473. https://doi.org/10.1172/JCI10842

Kucharska-Newton, A. M., Harald, K., Rosamond, W. D., Rose, K. M., Rea, T. D., & Salomaa, V. (2011). Socioeconomic indicators and the risk of acute coronary heart disease events: comparison of population-based data from the United States and Finland. *Annals of Epidemiology*, *21*(8), 572–579. https://doi.org/10.1016/J.ANNEPIDEM.2011.04.006

Leturque, A., Brot-Laroche, E., le Gall, M., Stolarczyk, E., & Tobin, V. (2005). The role of GLUT2 in dietary sugar handling. *Journal of Physiology and Biochemistry*, *61*(4), 529–537. https://doi.org/10.1007/BF03168378

Li, T., Chen, K., Liu, G., Huang, L. P., Chen, L., Wang, Q. W., Hu, C. L., & Hou, L. J. (2017). Calorie restriction prevents the development of insulin resistance and impaired lipid metabolism in gestational diabetes offspring. *Pediatric Research*, *81*(4), 663–671. https://doi.org/10.1038/PR.2016.273

Lowe, D. A., Wu, N., Rohdin-Bibby, L., Moore, A. H., Kelly, N., Liu, Y. E., Philip, E., Vittinghoff, E., Heymsfield, S. B., Olgin, J. E., Shepherd, J. A., & Weiss, E. J. (2020). Effects of Time-Restricted Eating on Weight Loss and Other Metabolic Parameters in Women and Men With Overweight and Obesity: The TREAT Randomized Clinical Trial. *JAMA Internal Medicine*, *180*(11), 1. https://doi.org/10.1001/JAMAINTERNMED.2020.4153

Lowrey, P. L., & Takahashi, J. S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annual Review of Genomics and Human Genetics*, *5*, 407–441. https://doi.org/10.1146/ANNUREV.GENOM.5.061903.175925

Meneton, P., Kesse-Guyot, E., Méjean, C., Fezeu, L., Galan, P., Hercberg, S., & Ménard, J. (2015). Unemployment is associated with high cardiovascular event rate and increased all-cause mortality in middle-aged socially privileged individuals. *International Archives of Occupational and Environmental Health*, *88*(6), 707–716. https://doi.org/10.1007/S00420-014-0997-7

Mezhnina, V., & Kondratov, R. (2020a). Regulation of glucose homeostasis by calorie restriction and periodic fasting. *Aging (Albany NY)*, *12*(23), 23422. https://doi.org/10.18632/AGING.104217

Mezhnina, V., & Kondratov, R. (2020b). Regulation of glucose homeostasis by calorie restriction and periodic fasting. *Aging (Albany NY)*, *12*(23), 23422. https://doi.org/10.18632/AGING.104217

Mitchell, S. J., Madrigal-Matute, J., Scheibye-Knudsen, M., Fang, E., Aon, M., González-Reyes, J. A., Cortassa, S., Kaushik, S., Gonzalez-Freire, M., Patel, B., Wahl, D., Ali, A., Calvo-Rubio, M., Burón, M. I., Guiterrez, V., Ward, T. M., Palacios, H. H., Cai, H., Frederick, D. W., … de Cabo, R. (2016). Effects of Sex, Strain, and Energy Intake on Hallmarks of Aging in Mice. *Cell Metabolism*, *23*(6), 1093. https://doi.org/10.1016/J.CMET.2016.05.027

Pancreas and insulin: An Overview - MyDr.com.au. (n.d.). Retrieved April 18, 2022, from https://www.mydr.com.au/pancreas-and-insulin/

Petersen, K. F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D. L., DiPietro, L., Cline, G. W., & Shulman, G. I. (2003). Mitochondrial Dysfunction in the Elderly: Possible Role in Insulin Resistance. *Science (New York, N.Y.)*, *300*(5622), 1140. https://doi.org/10.1126/SCIENCE.1082889

Powell-Wiley, T. M., Moore, K., Allen, N., Block, R., Evenson, K. R., Mujahid, M., & Diez Roux, A. v. (2017). Associations of Neighborhood Crime and Safety and With Changes in Body Mass Index and Waist Circumference: The Multi-Ethnic Study of Atherosclerosis. *American Journal of Epidemiology*, *186*(3), 280–288. https://doi.org/10.1093/AJE/KWX082

Roberts, S. B., Fuss, P. J., Greenberg, A. S., McCrory, M. A., Bouchard, T. J., Saltzman, E., & Neale, M. C. (2009). Genetic and environmental influences on factors associated with cardiovascular disease and the metabolic syndrome. *Journal of Lipid Research*, *50*, 1917–1926. https://doi.org/10.1194/jlr.P900033-JLR200

Röder, P. v., Wu, B., Liu, Y., & Han, W. (2016a). Pancreatic regulation of glucose homeostasis. *Experimental & Molecular Medicine 2016 48:3*, *48*(3), e219–e219. https://doi.org/10.1038/emm.2016.6

Röder, P. v., Wu, B., Liu, Y., & Han, W. (2016b). Pancreatic regulation of glucose homeostasis. *Experimental & Molecular Medicine 2016 48:3*, *48*(3), e219–e219. https://doi.org/10.1038/emm.2016.6

Röder, P. v., Wu, B., Liu, Y., & Han, W. (2016c). Pancreatic regulation of glucose homeostasis. *Experimental & Molecular Medicine 2016 48:3*, *48*(3), e219–e219. https://doi.org/10.1038/emm.2016.6

Roux, A. V. D. (2003). Residential Environments and Cardiovascular Risk. In *Journal of Urban Health: Bulletin of the New York Academy of Medicine* (Vol. 80, Issue 4).

Saini, V. (2010). Molecular mechanisms of insulin resistance in type 2 diabetes mellitus. *World Journal of Diabetes*, *1*(3), 68. https://doi.org/10.4239/WJD.V1.I3.68

Salgin, B., Ong, K. K., Thankamony, A., Emmett, P., Wareham, N. J., & Dunger, D. B. (2012). Higher fasting plasma free fatty acid levels are associated with lower insulin secretion in children and adults and a higher incidence of type 2 diabetes. *The Journal of Clinical Endocrinology and Metabolism*, *97*(9), 3302–3309. https://doi.org/10.1210/JC.2012-1428

Scarlett, J. M., & Schwartz, M. W. (2015a). Gut-brain mechanisms controlling glucose homeostasis. *F1000Prime Reports*, *7*. https://doi.org/10.12703/P7-12

Scarlett, J. M., & Schwartz, M. W. (2015b). Gut-brain mechanisms controlling glucose homeostasis. *F1000Prime Reports*, *7*. https://doi.org/10.12703/P7-12

Schenk, S., Mccurdy, C. E., Philp, A., Chen, M. Z., Holliday, M. J., Bandyopadhyay, G. K., Osborn, O., Baar, K., & Olefsky, J. M. (2011). Sirt1 enhances skeletal muscle insulin sensitivity in mice during caloric restriction. *The Journal of Clinical Investigation*, *121*. https://doi.org/10.1172/JCI58554DS1

Schultz, W. M., Kelli, H. M., Lisko, J. C., Varghese, T., Shen, J., Sandesara, P., Quyyumi, A. A., Taylor, H. A., Gulati, M., Harold, J. G., Mieres, J. H., Ferdinand, K. C., Mensah, G. A., & Sperling, L. S. (2018). Socioeconomic Status and Cardiovascular Outcomes. *Circulation*, *137*(20), 2166–2178. https://doi.org/10.1161/CIRCULATIONAHA.117.029652

Spinelli, R., Parrillo, L., Longo, M., Florese, P., Desiderio, A., Zatterale, F., Miele, C., Raciti, G. A., & Beguinot, F. (2020). Molecular basis of ageing in chronic metabolic diseases. *Journal of Endocrinological Investigation 2020 43:10*, *43*(10), 1373–1389. https://doi.org/10.1007/S40618-020-01255-Z

Study links metabolic syndrome to higher cardiovascular risk in patients with psoriasis | National Institutes of Health (NIH). (n.d.). Retrieved April 17, 2022, from https://www.nih.gov/news-events/news-releases/study-links-metabolic-syndrome-higher-cardiovascular-risk-patients-psoriasis

van der Zijl, N. J., Goossens, G. H., Moors, C. C. M., van Raalte, D. H., Muskiet, M. H. A., Pouwels, P. J. W., Blaak, E. E., & Diamant, M. (2011). Ectopic fat storage in

the pancreas, liver, and abdominal fat depots: impact on β-cell function in individuals with impaired glucose metabolism. *The Journal of Clinical Endocrinology and Metabolism*, *96*(2), 459–467. https://doi.org/10.1210/JC.2010-1722

Velingkaar, N., Mezhnina, V., Poe, A., & Kondratov, R. v. (2021). Two-meal caloric restriction induces 12-hour rhythms and improves glucose homeostasis. *FASEB Journal*, *35*(2). https://doi.org/10.1096/FJ.202002470R

Winkleby, M. A., Jatulis, D. E., Frank, E., & Fortmann, S. P. (1992). Socioeconomic status and health: how education, income, and occupation contribute to risk factors for cardiovascular disease. *American Journal of Public Health*, *82*(6), 816–820. https://doi.org/10.2105/AJPH.82.6.816

Woo, S., 1, K., Kim, H. J., 2, I. D., Min, K., Lee, H., Lee, S.-H., Kim, S., Kim, J. S., & Oh Id, B. (2021). *The relationship between smoking cigarettes and metabolic syndrome: A cross-sectional study with non-single residents of Seoul under 40 years old*. https://doi.org/10.1371/journal.pone.0256257

Ximenes, H. M. A., Hirata, A. E., Rocha, M. S., Curi, R., & Carpinelli, A. R. (2007). Propionate inhibits glucose-induced insulin secretion in isolated rat pancreatic islets. *Cell Biochemistry and Function*, *25*(2), 173–178. https://doi.org/10.1002/CBF.1297